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Defining the Role of Autophagy Kinase ULK1 Signaling in Therapeutic Response of Tuberous Sclerosis Complex to mTOR Inhibitors

PRINCIPAL INVESTIGATOR:

Reuben J. Shaw, Ph.D.

CONTRACTING ORGANIZATION:

The Salk Institute for Biological Studies
La Jolla, California 92037

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Shaw, Reuben J.

email: shaw@salk.edu

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14. ABSTRACT
The Tuberous Sclerosis Complex tumor suppressors are known to be critical negative regulators of the mTORC1 kinase complex that controls cell growth and autophagy. Our laboratory and others have recently decoded a major conserved route that mTORC1 uses to control autophagy. These studies demonstrate that mTORC1 inactivates another kinase complex composed of the autophagy kinase ULK1 and its associated subunits. One prediction of these findings is that in cells and tumors with TSC mutations and hyperactive mTOR, the ULK1 complex – and the process of autophagy – will be suppressed. There were two major aims for this funding period: 1) to further develop antibodies and reagents to readout ULK1-activity and substrate phosphorylation to see how well they act as biomarkers of mTOR inhibition, and 2) to further explore use of novel small molecule inhibitors of ULK1 to synergize with mTOR inhibitors to induce cell death.

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INTRODUCTION

The Tuberous Sclerosis Complex tumor suppressors are known to be critical negative regulators of the mTORC1 kinase complex that controls cell growth and autophagy. Our laboratory and others have recently decoded a major conserved route that mTORC1 uses to control autophagy. These studies demonstrate that mTORC1 inactivates another kinase complex composed of the autophagy kinase ULK1 and its associated subunits. One prediction of these findings is that in cells and tumors with TSC mutations and hyperactive mTOR, the ULK1 complex – and the process of autophagy – will be suppressed. There were two major aims for this funding period: 1) to further develop antibodies and reagents to readout ULK1-activity and substrate phosphorylation to see how well they act as biomarkers of mTOR inhibition, and 2) to further explore use of novel small molecule inhibitors of ULK1 to synergize with mTOR inhibitors to induce cell death.

BODY

As Task 1 was accomplished already in year 1 of the funding, in this second year we made significant additional progress on Task 2, 3, and 5. Task 2 was aimed at developing reagents to study ULK1 activity and function, and examine whether they are induced by mTOR inhibitors as we hypothesized. Task 2a was aimed at further identifying ULK1 substrates in vivo whose induction of phosphorylation by ULK1 may serve as functional biomarkers for mTOR inhibition in cells.

Figure 1. In vivo phosphorylation sites were defined in the ULK1-binding partner ATG13 when it was co-expressed with wild-type (WT) or kinase inactive (KI) ULK1 in HEK293T cells. We identified one specific serine in ATG13 – Ser389 that match the optimal ULK1 substrate consensus (LxxpSVxx), and are helping develop antibodies against this site.

Here we have made significant advances, identifying a number of additional direct ULK1 substrates that will be further summarized in the manuscript we have recently written up about ULK1 substrates which is currently under review at the journal Molecular Cell. As seen in Figure 1 for the ULK1-binding protein ATG13[1], only one specific serine amidst the many serines in the protein can serve as a highly phosphorylated direct ULK1-phosphorylation sites. The sequence of these sites also conforms to the optimal substrate motif identified above (LxxpSVxx). Task 2 also related to us examining the ULK1-dependency of the sites we discovered so we next examined whether we could detect a mobility shift in total ATG13 protein resolved on an SDS-PAGE gel, which might be indicative of ULK1-dependent phosphorylation. To most rigorously test this possibility, we examined Atg13 protein from wild-type and genetically knockout fibroblasts for Ulk1 and its close family member Ulk2[2] and combined treatment of such cells with starvation media (EBSS) or mTOR catalytic inhibitors (INK128, AZD8055), with or without use of our new tool compound ULK1 inhibitor 6-965 (see Figure 2, left).

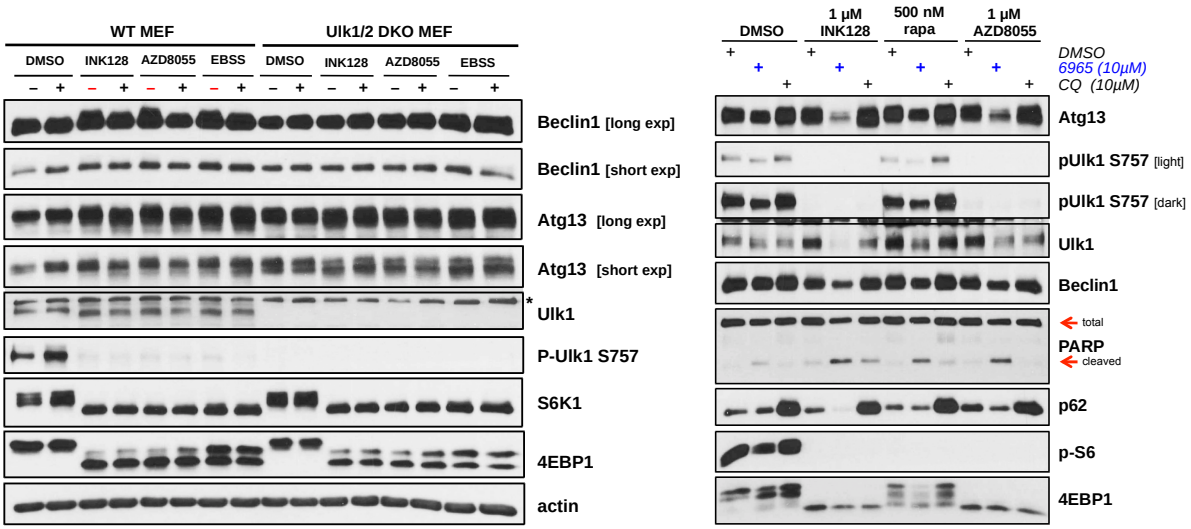

Figure 2. Examination of ULK1 targets as biomarkers of mTOR inhibition, and validation that our novel compound 6-965 acts as an ULK1 inhibitor. (**left**) Wild-type or *Ulk1/Ulk2* double knockout mouse embryonic fibroblasts (MEFs) (Cheong et al., 2011) were treated with fresh media (Dulbecco's modified eagle medium containing [DMEM] 10% fetal bovine serum [FBS]) containing 1 μM INK128, 1 μM AZD8055, or DMSO or with starvation media (Earle's balanced salt solution [EBSS] in the presence or absence of 10 μM 6965. Cellular lysates were isolated after 1h of treatment and immunoblotted with indicated antibodies. (**right**) A549 cells were treated with DMSO, 1 μM INK128, 500 nM rapamycin, or 1 μM AZD8055 in the presence or absence of 10 μM 6965 or 10 μM chloroquine (CQ). Cells were treated for 48 hours and immunoblotted with the indicated antibodies.

As seen in in the left panel of Figure 2, the mTOR inhibitors induced a modest but reproducible mobility shift in the Beclin1 and Atg13 proteins, indicative of increased phosphorylation. This increased mobility was suppressed by treatment with our ULK1 tool compound (+ lanes). No bandshift was seen in the ULK1/2 DKO MEFs, indicating that these mTOR inhibitor induced biochemical events are blocked by genetic or pharmacological inhibition of ULK1.

Next we connected these observations towards the main goal of Task 3 and Task 5: to examine the use of ULK1 inhibitors as a therapeutic to combine with mTOR inhibitors. In these tasks, we proposed to test whether inhibition of ULK1 can convert cytostatic effects of mTOR inhibitors into cytotoxic effects. Now that we have a tool compound, Compound 6 (6-695) that shows excellent catalytic inhibition towards ULK1 (see last year's report), we can perform these experiments. As seen in the A549 lung cancer cell line in the right hand panel of Figure 2, we examined whether 18h treatment with mTOR inhibitors with or without ULK1 inhibitor triggered caspase activation indicative of cell death. Indeed the extent of mTOR inhibition from INK128 vs AZD8055 vs rapamycin was proportional to the extent of caspase-cleaved PARP (Figure 2). This effect was greatest with co-treatment with the ULK1 inhibitor than with chloroquine (CQ), which supports our model that mTOR inhibition creates a cell survival mechanism dependent on ULK1. An even more robust biomarker of ULK1 inhibition in the context of mTOR inhibition was the stability of Atg13 protein, as seen in the right panel of Figure 2. We therefore propose total Atg13 protein levels as a biomarker of ULK1 inhibitor and mTOR inhibitor synergy.

We extended these key goals of this grant by functionally testing the ability of our novel ULK1 inhibitor to induce cell death synergistically with these three different mTOR inhibitors (Figure 3). These findings indicate that our original hypothesis that ULK1 inhibitors will push growth arrested mTOR-inhibited cells into apoptosis holds true, at least in this cell line. We will now push ahead to test this in the context of TSC-deficiency as proposed for year 3 of this funding.

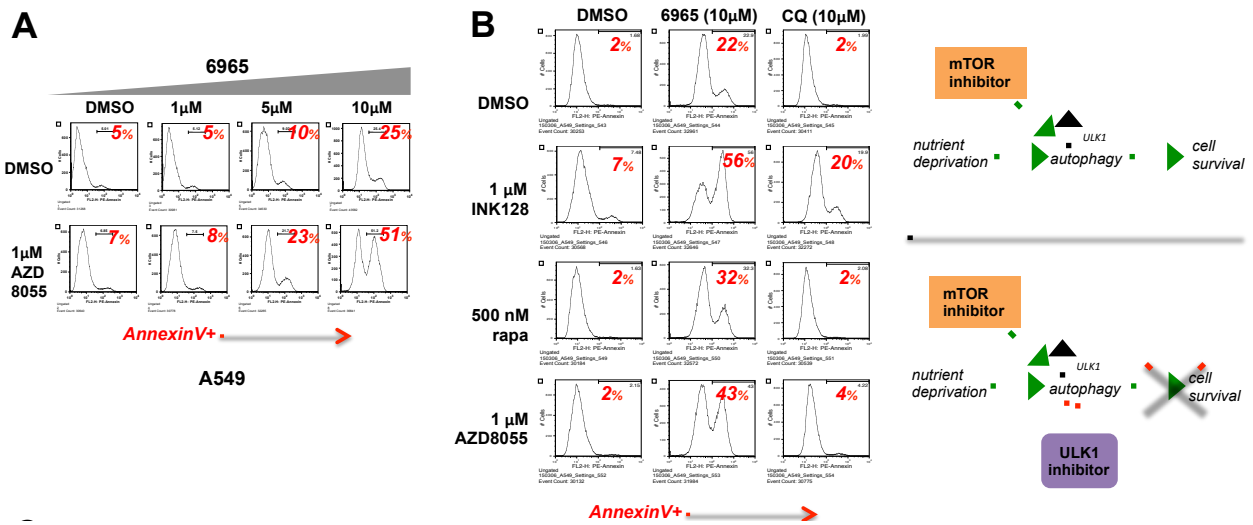

Figure 3. Treatment of A549 lung cancer cells with our novel ULK1 inhibitor compound 6-965 with or without mTOR inhibitors (AZD8055, INK128, rapamycin). (A) A549 human lung cancer cells were treated with the catalytic mTOR inhibitor AZD8055 (1 μM) or DMSO and increasing doses of 6965. Cells were treated for 72 hours and then collected, stained with PE-AnnexinV and quantified by FACS analysis. Red numbers indicate the percentage of AnnexinV- positive cells, representing cells actively undergoing apoptosis. (B) A549 cells were treated with DMSO, 1 μM INK128, 500 nM rapamycin, or 1 μM AZD8055 in the presence or absence of 10 μM 6965 or 10 μM chloroquine (CQ). Cells were treated for 48 hours and then collected, stained with PE-AnnexinV and quantified by FACS analysis.

KEY RESEARCH ACCOMPLISHMENTS

- identification of ULK1 phosphorylation sites in 4 protein to act as biomarkers in vivo
- identification of a ULK1 inhibitor tool compound that synergizes with mTOR inhibitors to trigger death of cancer cells

REPORTABLE OUTCOMES

Manuscript submitted to Molecular Cell and currently under review.

CONCLUSION

Our findings during this second year of funding have been very fruitful, accomplishing multiple Tasks as proposed in the Statement of Work of the grant. In particular, we have identified Atg13 as a ULK1 substrate in vivo and how shown that Atg13 total protein levels are an excellent biomarker of effective ULK1 and mTOR inhibition. Given the widespread use of mTOR inhibitors including rapalogs to treat Tuberous Sclerosis Complex, the identified of new biomarkers for TSC treatment is particularly exciting. In addition, we have further characterized our small molecule ATP-competitive inhibitors of ULK1 and our Compound #6-965 behaves as an excellent on target inhibitor of ULK1 that synergizes converts the cytostatic growth arrest of cells starved of nutrients into a cytotoxic cell death response. We are now testing further how well this compound synergizes with mTOR catalytic inhibitor in TSC-deficient cancer cell lines and ultimately mouse models, consistent with the aims of the grant proposal here.

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